



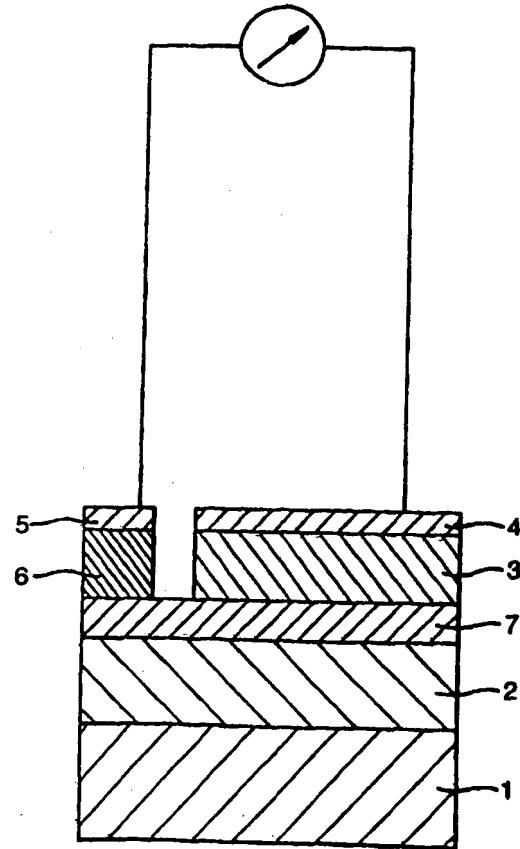
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(54) Title: PROTEIN SENSOR

(57) Abstract

There is described an apparatus for detecting the level of protein in a sample, especially for determining the level of protein in a dust sample. The apparatus uses two sequential reaction zones, in the first of which protein is subjected to degradation by proteolytic enzyme liberating amino acids, followed by detection of amino acids using a second amino acid oxidase enzyme whose level of activity can be detected by detecting enzymic reaction products. Amino acid oxidase activity is preferably followed by detecting the level of hydrogen peroxide, preferably electrochemically using an electrode (4) having a catalytic surface (3). The electrode surface may be provided by depositing an ink composition comprising a mixture of amino acid oxidase enzyme and conductive particles with catalytic surfaces onto a conductive substrate. Protease enzyme is immobilised on a membrane (1) and barrier layers (7 and 2) prevent contact between protease and amino acid oxidase but allow permeation by amino acids in solution.



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PROTEIN SENSOR

The present invention relates to an apparatus for detecting the level of protein in a sample, especially a 5 dust sample for instance collected from a surface or from the air. The sensor apparatus uses two sequentian reaction zones in the first of which protein is subjected to degradation by proteolytic enzyme liberating amino acids, followed by detection of amino acids using a second, amino 10 acid oxidase enzyme, whose level of activity can be detected by detecting enzymic reaction products. Preferably the level of activity of the amino acid oxidase enzyme is determined by detecting the level of hydrogen peroxide electrochemically, using an electrode based on 15 rhodinised carbon.

The present inventors propose that airborne protein concentrations can serve as an indicator of home hygiene. The sources of protein, found (in the air) within typical homes include: human and animal skin cells, allergens 20 (e.g. pollen, dust mites and their faecal particles), fungi, bacteria and viruses. The last point is of particular concern for asthma and allergic rhinitis sufferers, where sensitivity to dust mites (and their faecal pellets) has been shown to be a major contributory 25 factor in the development of these maladies. A considerable number of people (approximately 40 million) in the USA are believed to be affected by either or both of these diseases. Workers have considered measuring these various elements, but no-one appears to have thought of 30 measuring general protein as an indicator of the overall level of contamination.

Despite the large research effort that has gone into the development of biosensors over the last few decades, no biosensor systems aimed at "impure" protein concentration 35 detection have been described. In addition, very few biosensors capable of detecting analytes in the gas phase have been described. One notable exception to this was the

work of Dennison et al (1995) (Dennison, M.J., Hall, J., & Turner A.P.F., 1995 "A gas phase microsensor for monitoring phenol vapour at ppb levels" *Anal. Chem.* 67, 3922-27). The authors described the use of a gel matrix containing enzymes, suitable for detecting the presence of analytes in the gas phase. The gel was packaged into a biosensor format and was capable of detecting analytes (such as phenol) at low concentrations.

A number of reports describing the development of biosensors for the detection of amino acids (the constituent of proteins), have been published. These include both electrochemical and optical based devices. Typical of these reports is the paper by Kacaniklic et al (Kacaniklic, V., Johansson, K., Marko-Barga, G., Gorton, L., Jonsson-Pettersson, G., Csoregi, E. (1994). Amperometric biosensors for detection of L- and D- amino acids based on coimmobilised peroxidase and L- and D- amino oxidases in carbon paste electrodes. *Electroanalysis* 6, 381-390), where a bienzyme (peroxidase and amino acid oxidase) electrode was constructed and used to detect free amino acids in a buffer solution. A flow injection analysis system was used, permitting multiple measurements to be made. Using this system, the authors were able to detect all twenty of the most common L-amino acids.

However, most of these biosensors detect the amino acid in its free state. The only report (to the knowledge of the inventors) where a preparation stage occurs, is described in the publication by Male et al, (Male, K.B., Luong, J.H.T., Gibbs, B., Konishi, Y. (1993). An improved FIA biosensor for the determination of aspartame in dietary food products. *Applied Biochemistry and Biotechnology* 38, 189-201). A flow injection analysis system was developed, with the aim of detecting aspartame (L-aspartyl-L-phenylalanine methyl ester). This compound is rapidly replacing saccharin as a low calorie sweetener in many food products. Hence a method to accurately determine the concentration of aspartame in food products is highly

desirable. The system consisted of an enzyme column of immobilised pronase and a L-amino acid oxidase electrode connected in series. The dipeptide bond of aspartame was cleaved by the immobilised pronase. This action released 5 free phenylalanine into the running buffer, which in turn was detected at the amino acid oxidase electrode. Male et al includes an analysis of the selectivity of the amino acid oxidase enzyme to various amino acids and determined that the oxidase was sensitive to amino acids other than 10 the amino acid of interest, in that case phenylalanine. Indeed the enzyme used gave results varying from 0 (for aspartate, glutamate, glycine, serine, proline and threonine) which was unmeasurable up to tyrosine, which had a response of 183 based on 100 as the response to 15 phenylalanine. The amino acid oxidase is type L-amino acid oxidase type IV (E.C.1.4.3.2). The pronase was from *Streptomyces griseus*, available commercially from Boehringer Mannheim. Its activity against peptide bond of other compounds other than aspartame was not investigated. 20 However tests were carried out on various dietary food products including Jello (believed to be a gelatine-containing dessert), vanilla pudding and hot chocolate. Especially for the latter compound the results for the estimated level of aspartame was significantly different to 25 the manufacturer reported level.

The sensor developed by Male et al is intended to determine the level of a specific compound formed of two amino acids (aspartame) in a sample which may contain protein. Consequently it would be expected that for 30 optimum performance, the enzymic reactions as a whole should be specific so that the end product amino acids are not derived from components of the food product under test other than aspartame.

Another sensor is that described in JP4348270 which 35 describes a sensor comprising a baseplate, two insulating films, a pair of electrodes, an instillator, and current or voltage measuring unit. The first film is an aminoacid

oxidase fixed porous polyacrylonitrile film, while the second film is piled on the first film, and is a proteolytic enzyme fixed porous polyacrylonitrile film. A pair of electrodes is used to detect produced H₂O₂ on the 5 first film. The instillator is used to supply water or aqueous solution to the second film. When pollen adheres to the second film, antigen proteins in the pollen dissolve into the liquid on the second film and are decomposed by the proteolytic enzyme to aminoacids. The aminoacids are 10 decomposed by aminoacid oxidase to H₂O₂, keto acid, and ammonia. The H₂O₂ produced is measured by H₂O₂ detecting electrodes as current or voltage changes.

The objective of the present invention is to determine the level of total protein in a sample and is thus required 15 to be non specific. The device is intended for use in a domestic environment and must consequently be safe to use in the presence of pets and children. The device should be simple to use with the minimum amount of manual manipulation. Disposal of spent sensors should be easy and 20 safe. The speed of response of the device must be over an acceptable time span and the device should be affordable and thus use simple fabrication techniques.

The present invention comprises a new apparatus which is a sensor for the detection of the level of protein in a 25 sample and comprises:

a first reaction zone which contains a non-specific protease enzyme;

a second reaction zone containing a non-specific amino acid oxidase enzyme included at an electrode surface, and 30 at least one other electrode capable of being in electrical contact via a liquid electrolyte with the first electrode;

a conduit for passage of fluid from the first reaction zone to the second reaction zone; and

separating means in the conduit for preventing passage 35 of protease enzyme from the first reaction zone to the second reaction zone and for preventing passage of amino acid oxidase enzyme from the second reaction zone to the

first reaction zone, said means allowing for the diffusion of amino acids.

It should be appreciated that the apparatus of the invention may be provided in kit form consisting of 5 component parts incorporating cooperating means. These enable the component parts to be fitted together to form the apparatus of the invention. The reaction zones and the electrode assemblies may be provided separately and assembled prior to protein detection.

As mentioned above, the apparatus of the invention is of particular value for determining the level of protein in a sample which contains a mixture of proteins, of unknown type and level. The sample is preferably of dust, especially house dust. The sample may be collected from 15 air, the primary source of airborne protein causing the above mentioned health problems. Alternatively the sample may be of dust collected from a surface, which may be a good indicator of the level of airborne dust.

Where the sample is of airborne dust, it may be 20 collected by a preliminary step in which a predetermined amount of air is drawn through a filter having apertures of an appropriate size to collect dust, whereby the sample is collected on the pad surface from which it may either be recovered into a liquid or gel reaction mixture apparatus 25 or the filter may form the reaction zone in which the proteolysis takes place, by having a liquid or gel applied to it to create the digestion mixture. Alternatively the sample may be collected directly into a liquid or gel by drawing a predetermined amount of air into a vessel where 30 it is contacted with a liquid or gel whereby suspended articles are retained in the liquid or impacted onto a gel or other solid medium that can support enzyme activity.

Where the sample is dust collected from a surface then collection may be carried out by the use of a swab, usually 35 a swab imbued with a fixed amount of liquid or gel, which is used to wipe a predetermined area of the surface. The collected dust on the swab is then recovered or collected

onto a gel (e.g. adhesive surface) preferably containing the protease enzyme.

Regardless of the sample collection procedure, it is important that the volume of the digestion mixture used in 5 the first reaction zone of the apparatus and which contains the sample collected from predetermined air volume or surface area is known, since it is probable that the initial reaction in the first reaction zone will not be carried out to completion. Thus the rate of the 10 proteolytic reaction will ultimately be determined which can be correlated with the concentration of protein in the original sample.

The apparatus may comprise a dust collector. This component may be suitable for collecting airborne dust, and 15 may include means for drawing air through a filter having apertures of suitable size for collecting airborne dust particles. Alternatively the collector may be more suitable for collecting dust from a surface. It may be a wet or a dry pad. The collector may comprise the first 20 reaction zone comprising protease enzyme, where such reaction zone has suitable properties to act as a collector, for instance where it comprises a porous material such as a membrane. Alternatively the collector should be contacted with the first reaction zone to allow 25 passage of protein from the sample to the first reaction zone where protein digestion by the protease enzyme takes place. A collector which is a generally sheet form filter, may be in direct face to face contact with a membrane carrying immobilised protease enzyme, optionally with a permeable protective layer located between the sheets for protection. A solution of protein can thus flow directly 30 from the dust collector to the first reaction zone.

The protease enzyme must be non specific, that is it should act on substantially any protein, that is cleaving 35 any of the peptide bonds of a protein at substantially the same rate or at least at a rate which is within a similar order of magnitude. Usually such enzymes cleave terminal

amino acids from linear polypeptides. One suitable protease which the present inventors have determined is unusually non-specific is type XIV protease from *Streptomyces griseus*. This enzyme is alternatively known
5 as PronaseE.

It may be possible to use a protease enzyme which is more specific, provided that the correlation between the final result obtained by the sensor and the level of total protein in the sample is good. Thus it may be possible to
10 carry out empirical tests to find a relatively specific protease which nevertheless results in a level of amino acid in the reaction mixture of the proteolytic reaction which correlates well with total protein in a wide range of household dust samples. Alternatively where the sample
15 contains mixed proteins which are of a non varying range of types, the level of the final reaction product should correlate well with total protein. Thus the level of total protein can be determined by use of appropriate calibration procedures.

20 Alternatively, where the protease enzyme is relatively specific in its reactivity, so that it may favour generation of certain free amino acids over others, the specificity may be outweighed by an inverse specificity in the amino acid oxidase enzyme.

25 The protease enzyme is preferably immobilised in the first reaction zone of the new apparatus. Conventional methods for immobilising the enzyme may be used. Such methods generate covalent bonds between side groups of amino acid residues of the protein and activated pendant groups on substrate surfaces. The substrate may, for instance, be a membrane or beads in a column. A suitable substrate is Immunodyne (trade mark) membrane, for instance Immunodyne ABC which has activated pendant groups which react with nucleophilic groups, usually amine groups, on
30 the enzyme. Where the apparatus is a flow injection system, the enzyme is preferably immobilised on beads in a column through which liquid digestion mixture flows.

Immobilisation of the protease enzyme in the first reaction zone is generally carried out by covalently bonding the enzyme to a reactive membrane substrate such as Immunodyne. Appropriate tests can be carried out to ensure
5 that the immobilisation reaction does not reduce the activity of the enzyme. Where the substrate has active pendant groups, to ensure that no active groups remain after immobilisation of the enzyme, the results is preferably subjected to a deactivation step in which excess
10 of a small molecule having a counter reactive group is contacted with the substrate bearing immobilised enzyme.

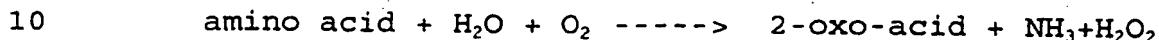
The amino acid oxidase should preferably be relatively non specific. Proteins are formed from, and degraded to, L-amino acids. It is preferred for the amino acid oxidase
15 enzyme not to react with D-amino acids, which may be present in some samples. The present inventors have determined that a suitable enzyme is L-amino acid oxidase EC 1.4.3.2.

In the apparatus it is important that the two enzymes
20 are not contacted with one another. The reason for this is that the amino acid oxidase enzyme may be susceptible to proteolysis by the protease enzyme. The enzymes should thus be physically separated from one another. Where the apparatus is a flow injection analysis type system, in
25 which there is a first reaction zone of a sensor, and the second stage is carried out in a different reaction zone of the sensor, this physical separation of the enzymes will be achieved by adequate immobilisation of the protease enzyme and avoiding reverse flow of reaction mixture from the
30 second reaction volume to the first reaction volume. Where it is impossible to prevent reverse flow of liquid from the zone in which the amino acid oxidase reaction is taking place to the zone in which the proteolysis is taking place, provided both enzymes are adequately immobilised and are
35 physically separated from one another no further separation means should be necessary. However where immobilisation of one or both of the enzymes may be incomplete, or may

deteriorate during the process, it is desirable to include a physical barrier, for instance a membrane which would prevent passage of either of the enzymes between the two reaction zones.

- 5 Amino acid oxidase operates by oxidising the substrate (amino acid) in the presence of molecular oxygen, producing a 2-oxo-acid and hydrogen peroxide.

amino acid oxidase



There are several ways which can be used in the apparatus of the invention for following this reaction using biosensors. One approach is to monitor the 15 consumption of oxygen, resulting from the enzyme reaction. A difficulty with this technique is that the result can be significantly affected by local changes in oxygen concentrations caused by extraneous factors which are difficult to control.

20 A second method for following the amino acid oxidase reaction to use an ammonia electrode. Again, the results of these methods may be affected by other factors which change the pH or the presence of other amines which may affect the ammonia electrode.

25 The third approach for monitoring the amino acid oxidase reaction is the use of mediators (reference: Dicks, J.M., Aston, W.J., Davis, G. & Turner A.P.F. (1986). Mediated amperometric biosensors for D-galactose, glycolate and L-amino acids based on a ferrocene-modified carbon 30 paste electrode. *Analytica Chimica Acta*, 182, 103-112. These compounds act as electron acceptors or donors, coupled to the enzyme reaction. A wide range of mediators have been described in literature including, quinones, organic dyes and ferrocene based compounds. These 35 compounds can be used (for example) to replace oxygen as the electron acceptor. Hence, the drawbacks outlined above when using oxygen depletion can be largely eliminated. In

addition mediators offer advantages in terms of lower operating potential (reducing the effects of electrochemical interrefernts), a broader pH operating range and less dependence on the fluctuation local oxygen concentration.

The fourth and preferred technique for following the amino acid oxidase reaction, is to monitor the production of H₂O₂.

Monitoring H₂O₂, generated enzymatically, has been widely used in the field of biosensors. Hydrogen peroxide can be electrochemically decomposed, and the resulting current amperometrically determined using a suitable electrode array. This method of monitoring has been more widely used in the development of biosensors and a plethora of such devices have been described in the literature. Alternatively peroxide may be enzymically decomposed to generate a coloured reaction product.

Both in the past and today, the present applicant has been at the forefront of both fundamental research and development of commercial sensors based on H₂O₂. Such work has focused particularly on using transition metal (e.g. platinum, palladium and rhodium)/carbon base electrodes, to determine the rate of the catalysed reaction:

H₂O₂ -----> 2H⁺ + 2e⁻ + O₂. Electrodes formed from these materials display high current densities in the presence of H₂O₂, whilst maintaining a significant degree of selectivity. Previous work at Cranfield (Newman J.D., White, S.F., Tothill, I.E., Turner, A.P.F. (1995). Catalytic materials, membranes, and fabrication technologies suitable for the construction of amperometric biosensors. *Analytical Chemistry* 67, 4594-4599) had shown that the promoted rhodinised-carbon powder, designated MCA4 (MCA Ltd. Cambridge, UK), had superior qualities for biosensor operation compared to other metal/carbon electrodes. Principally, a higher current density response and improved selectivity. In addition MCA4 is supplied in a form suitable for inclusion in a screen printing ink.

The invention uses an electrode system to determine the electrochemical decomposition of hydrogen peroxide. Preferably the electrode system determines directly the rate of the reaction $H_2O_2 \rightarrow 2H^+ + 2e^- + O_2$.

5 Alternatively the decomposition of hydrogen peroxide may involve a coupled reaction, for instance an enzymic reaction, for instance using horseradish peroxidase as described in EP-A-0585113 or using NADH-peroxidase.

In the preferred embodiment of the invention, using a catalytic degradation of hydrogen peroxide the amino acid oxidase is incorporated in immobilised form at the electrode surface. Thus amino acid oxidase acts on the amino acid generated in the first reaction zone of the apparatus to generate hydrogen peroxide which is immediately catalytically decomposed at the electrode surface in the second reaction zone generating a current which is measured to determine the rate of the reaction.

10 Measuring the extent of oxidation allows determination of the maximum current reached, or the current after a predetermined period of time or measure the current over time until it falls to zero or a predetermined level based on the maximum reached as the datum point for reporting the level of amino acid and hence the amount of protein in the original sample.

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20 In the apparatus, the second reaction zone preferably includes the amino acid oxidase enzyme bound to a substrate. The substrate is, in a preferred embodiment, at the surface of an electrode, preferably a catalytic surface which catalyses the electrochemical degradation of hydrogen peroxide. The enzyme may be immobilised at the surface of such an electrode by covalently bonding it to particles which can subsequently be coated onto the electrode surface. It has been found that, however, the enzyme need not be covalently bonded to a particulate carrier but can merely be physically blended with other components of a coating composition for forming the catalytic surface. In a preferred embodiment, the enzyme is blended with

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rhodinised carbon particles described by Newman et al (op.cit). The blend, as a dispersion in a liquid vehicle can be printed on loan electrode substrate and the liquid vehicle removed by evaporation. The coating may 5 subsequently be provided with a barrier coating permeable to amino acids, which prevents the enzyme being removed by liquid during use.

The amino acid oxidase enzyme is combined with conductive particles having a catalytic surface suitable 10 for catalysing the composition of hydrogen peroxide into a composition comprising a liquid vehicle. This is provided a printable liquid composition comprising a liquid vehicle and, suspended in the liquid vehicle, electrically conductive particles having a catalytic surface for 15 catalysing decomposition of hydrogen peroxide and amino acid oxidase enzyme.

The liquid composition is suitable for printing onto an electrode substrate. Generally it will comprise a thickener and/or suspending agent to maintain the disbursed 20 conductive particles and enzyme in suspension. The liquid vehicle may, for instance, be aqueous or based on a non-aqueous organic solvent. Preferably, for optimum maintenance of enzymic activity in the final product, the liquid vehicle is aqueous.

25 The electrode component comprises a substrate which is an electrically conductive solid having on at least one surface a coating comprising conductive particles in electrical contact with the substrate and amino acid oxidase enzyme, comprising further an amino acid oxidase reaction zone abutted by both enzyme and conductive 30 particles. This electrode component may be incorporated into apparatus which includes electrical connections for connecting the electrode substrate to an amperometric detector, connected further to a counter electrode. 35 Preferably the apparatus includes electrolyte providing electrical contact between the electrode and the counter electrode.

Preferably the apparatus is used to determine the level of protein in a sample and comprises therefore an amino acid generation reaction zone and a conduit for supply of product mixture from the amino acid generation reaction zone to the amino acid oxidase reaction zone.

The conduit for passage of fluid from the first reaction zone to the second reaction zone may be any tube, channel or passage in which liquid flows from the first reaction zone to the second reaction zone, the barrier to enzyme passage being constituted by complete immobilisation of the upstream enzyme, that is the protease enzyme and provision of continuous flow in the direction first reaction zone to second reaction zone. Alternatively the barrier means may be constituted by a physical shield which is permeable to low molecular weight compounds, such as amino acids, but which is impermeable to higher molecular weight compounds such as enzymes. The present inventor has discovered that a suitable shield is constituted by membrane substrate material such as that constituted by Immunodyne membranes. Where, as for Immunodyne membranes itself, there are reactive pendant groups on the membranes these are preferably blocked by a coreactive small molecule to render the membrane inert. This prevents those reactive groups binding to components of the reaction digestion product mixture during passage through the membrane.

In the apparatus of the invention the conditions under which protease activity is optimised may differ somewhat from those under which amino acid oxidase activity is optimised. For instance protease type XIV from *Streptomyces griseus* requires the presence of calcium ions. By contrast the preferred amino acid oxidase enzyme is optimised in the presence of a phosphate buffer. However calcium ions and phosphate ions generate a precipitate in aqueous admixture. Consequently the apparatus may include the addition to the enzymic reaction mixture from the first stage additional components for the reaction mixture for the second stage. Where the apparatus is a flow injection

system, the further components are injected into the flow downstream from the reaction zone where the first stage takes place. Where the two enzymic reactions are carried out in the same vessel, additional components are added to 5 that vessel after a predetermined period of time during which the first reaction zone is carried out, before the second step commences. Where the two enzymic reactions are each carried out in different vessels, the second vessel may be provided with the desired components prior to 10 addition of the first stage product mixture to the vessel.

It may be desirable for the first reaction zone of the apparatus to subject the digestion mixture to agitation, in order to maximise contact of the protease enzyme with protein in the digestion mixture. Although the digestion 15 mixture may be subjected to raised temperature, preferably the reaction is carried out at ambient, that is around room temperature. The digestion may or may not be carried out to completion. Where it is not carried out to completion the length of the digestion step should be constant between 20 tests. The digestion is preferably carried out for a period in the range 1 minute to 1 day, preferably 10 minutes to 4 hours. Most conveniently the time is less than 30 minutes.

Where the protease enzyme is protease type XIV from 25 *Streptomyces griseus*, the digestion mixture preferably contains calcium ions in a concentration in the range of 0.5 mM to 10 mM, preferably in the range 1 to 5 mM. The pH is preferably in the range 6 to 8, more preferably above 7.

In the digestion step, the concentration of total 30 solid sample in the reaction mixture, of which all or, usually, at least 50% is protein, is generally in the range 0.1 to 10% by weight, preferably 1 to 5%. The enzyme should be present in an amount based on the weight of the reaction mixture in the range 0.5 to 20% by weight.

35 The amino acid oxidase enzymic reaction is, also, preferably carried out under ambient conditions of temperature. The pH should preferably be in the range 6 to

8, more preferably about 7. Other preferred components of the reaction mixture for the amino acid oxidase step are potassium chloride and buffer, for instance sodium phosphate buffer. Where additional components are added to
5 the reaction mixture following the reaction in reaction zone one, the level of dilution is generally in the range 1:2 to 1:5 preferably about 1:3. The period for the amino acid oxidase reaction is usually at least 10 sec. Preferably in the range 1 minute to 1 day, for instance in
10 the range 2 to 30 minutes.

Either or both enzymic reactions are carried out in a liquid reaction mixture or in gel reaction mixtures, where these can support the enzymic reactions. The use of gel media may make the apparatus easier to handle.

15 With regard to the electrode component of the device, conventional amperometric electrodes can be designed to operate using either a two electrode or three electrode system. In both systems there is a requirement for a stable reliable reference electrode to control the potential of the working electrode, usually by means of a potentiostat. The two electrode approach has the advantage of being simple and requiring less instrumentation. However it can suffer from a possible drawback. Generally the system operates by driving the current through both the
20 electrode interfaces. This can result in a situation whereby it is difficult to determine which of the electrode pair is primarily determining the current measured, or whether the current is determined by a balance between the two. However, with care, errors emanating from the two
25 electrode method can be eliminated.
30

The invention is further illustrated in the accompanying drawings in which:

Figure 1 is a schematic representation of the apparatus of the invention;

35 Figure 2 shows the electrode response of a peroxide electrode with immobilised amino acid oxidase before and

following contact with a control (no protein) sample [see details in Examples];

5 Figure 3 shows a electrode response before and after contact of the same electrode used in Figure 2 with the enzymic product mixture of protease enzyme on a sample of dust; and

10 Figure 4 shows a calibration curve for electrode response against amino acid concentration for the amino acid oxidase/peroxide electrode.

15 In Figure 1 there is shown a simplified schematic diagram of one embodiment of the apparatus of the invention. A filter component 1 comprises a substrate membrane, preferably formed of Immunodyne membrane, to which protease enzyme is immobilised. Dust is collected on the filter 1 by drawing air through the filter in a preliminary step prior to assembly with the other apparatus components. The digestion reaction mixture is completed by provision of a predetermined quantity of appropriate buffer to the filter, which provides a medium for the enzymic reaction to take place. Amino acids which are the reaction product of the proteolytic reaction taking place in first reaction zone comprised by filter 1 travel through physical separation means 2. Membrane 2 is a chemically inert membrane having apertures large enough to allow free 20 passage of amino acids therethrough whilst preventing passage of higher molecular weight components such as amino acid oxidase or protease enzymes.

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30 The catalytic surface 3 of an electrode is provided by coating the carbon pad 4 of an electrode with a water-borne printing ink comprising amino acid oxidase enzyme in physical admixture with suspended rhodinised carbon particles. A reference electrode 5 is coated with a silver/silver chloride ink coating 6. Both the coated electrodes are then dipped in a water-insoluble cellulose acetate which provides a physical barrier 7 preventing removal of surfaces 3 and 6 from the respective membranes

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and providing electrical contact through the electrolyte between the two electrodes.

Amino acids pass through the barrier 2 and the cellulose acetate layer 7 to the catalytic surface 3. Upon 5 contact of the amino acids with amino acid oxidase enzyme in an appropriate buffer which is located in the apertures in coating 7 and between the particles at the surface 3, hydrogen peroxide is produced as a product of the enzymic oxidation reaction. Hydrogen peroxide is electrochemically 10 degraded with rhodium as catalyst. The current which passes from electrode 4 to counter electrode 5 is proportional to the rate of hydrogen peroxide degradation which in turn is proportional to the rate of oxidation of amino acids which in turn is proportional to the 15 concentration of amino acid passing from the digestion step taking place in membrane 1. The amino acid concentration is in turn proportional to the rate of proteolytic reaction which, in turn, is proportional to the concentration of protein in filter 1.

20 The components of the apparatus and the levels of the various reagents, enzymes and catalysts used for a preferred embodiment of the invention is described in the following examples.

Example 1

25 Reaction Zone 1 - Digestion

Protein digestion was achieved using immobilised protease (Type XIV; from *Streptomyces griesus*; Pronase E), bound to Immunodyne ABC membrane (Pall BioSupport, Portsmouth, England). An important feature of this 30 membrane material are the covalent bonds it forms with the enzyme, via the functional groups. Immunodyne ABC is pre-activated to form covalent linkages with nucleophilic groups found on proteins and other macro molecules. Primary reactivity is with amine groups at alkaline pH.

35 The immobilisation process was carried out by spot wetting a protease solution (10 μ l of an enzyme solution comprising: 50mg protease in 100 μ l of 0.1M phosphate buffer

containing 0.1M potassium chloride) onto a strip of the membrane (10mm x 2mm). The solution was left to dry for 1.5 hours at room temperature (approximately 25°C). Following drying the membrane was blocked using 50mM TES (N-tris[hydroxymethyl]methyl-2-aminoethane-sulphonic acid) containing 5mM CaCl₂, pH 7.0. (adjusted using 10M NaOH). Normal practice is to use a protein compound for blocking processes. However, for this application it was felt that this may provide an alternative source for the detection system (considering it is protein which is being detected). TES buffer contains an amino compound that binds covalently to the membrane. It is an effective blocking compound for this application. The protease requires the presence of calcium ions in order to facilitate its reaction. Hence the inclusion of calcium ions. Obviously TES buffer could not be used for the immobilisation step, as the buffer would be in competition with the enzyme for available binding sites. Blocking was carried out by incubating the protein strip for 1 hour in the buffer solution. During this time the incubation solution was gently agitated. Following blocking the strips were left in buffer at 4°C, until required.

Example 2

Barrier Membrane

A second strip of Immunodyne ABC (10 x 15mm) was prepared by incubating in the TES solution for 2 hours. This second membrane forms the chemically inert protective barrier preventing passage of the protease to the reaction zone containing the enzyme amino acid oxidase (the biological component of the biosensor). Following blocking the membrane was left to dry in air at room temperature.

Example 3

Reaction Zone 2 - Amino Acid Oxidase Biosensor

The detection component was based on an amino acid oxidase biosensor, using a two electrode system; amperometric detection being the basis of the sensor.

The biosensor was constructed using screen printing techniques. A working electrode ink comprising: 10mg of L-amino acid oxidase (EC 1.4.3.2; obtained from crude dried venom of *Crotalus adamans*), 25mg of MCA4 (carbon/transition metal compound) and 100 μ l of phosphate buffer pH 7.0, was prepared and applied to the carbon pad (forming one end of an electrode). A second reference/counter electrode was printed using commercially available Ag/AgCl ink. Both electrodes were dip-coated with a cellulose acetate solution (1% cellulose acetate in acetone w/v). The purpose of this membrane was to retain the water soluble working electrode ink, whilst permitting flux of liberated amino acids to the sensor surface.

Example 4

15 Assembly and use of the apparatus on dust and pure mixed protein

All of the experiments were carried out by "sprinkling" 2mg of sample (either dust or Casilan 90 a commercially available source of pure protein) onto the protease membrane produced as in Example 1. An aliquot (40 μ l of TES buffer containing 5mM of CaCl₂) was deposited over the dust/membrane combination and left to incubate for 1 hour.

After this time, the Immunodyne barrier membrane produced as in Example 2 was placed over the electrode array and 75 μ l of 50mM TES with 5mM CaCl₂ and 0.1M KCl (which serves as the electrolyte) deposited on top. The electrode potential was set at 350mV. When a steady state electrochemical signal was achieved; the protease/sample membrane was placed on top of the blocking membrane, directly over the area of the working electrode. Increases in current were attributed to the electrochemical decomposition of hydrogen peroxide; a product of the amino acid oxidase reaction. Control experiments were carried out using the same arrangement with: 1, no sample present 2. sample with no immobilised protease. Figure 2 shows a typical response curve for a control sample whilst Figure 3 shows a typical response curve for a dust sample.

Table 1 depicts the response from thirteen samples. The first column shows the response (none) in the absence of sample and the second column depicts the response from samples. Only one sample failed to give a response.

5

TABLE 1

Electrode No.	Control	Current After Contact of Sample	Sample Type
1	- no response	707nA	Casilan
2	- no response	1540nA	Casilan
3	- no response	1894nA	Casilan
4	- no response	600nA	Dust
5	- no response	40nA	Dust
6	- no response	50nA	Dust
7	- no response	-	Dust
8	- no response	165nA	Dust
9	- no response	77nA	Dust
10	- no response	264nA	Dust
11	- no response	228nA	Dust
12	- no response	72nA	Dust
13	- no response	947nA	Dust

It is believed that improvement in the device will lead to the current in the response being proportional to the protein concentration in the digestion mixture.

Example 5Calibration of Electrode Response for Amino Acid Concentration

Solutions of varying concentrations of lencine in 50mM TES with 5mM CaCl₂, and 0.1M kcl were contacted with the amino acid oxidase sensor. Figure 4 shows the response of the electrode against the concentration of lencine and indicates a linear response between 0.25 and 8.0mM lencine. The response times were of the order of 30 seconds to achieve maximum current, for each concentration.

Example 6Dust Collection

Tests were carried out using alternative means of contacting a dust sample with protease membrane produced as in Example 1. As an alternative to Example 4, which sprinkles 2mg of dust sample directly onto the protease membrane, a second protease strip may be positioned over the dust sample on a first protease strip so that the dust which is consequently sandwiched between two strips. The conditions are otherwise the same as in Example 4.

As a second alternative, the dust is first deposited over a glass fibre filter pad, which is subsequently covered by the protease strip. The buffer for the protease reaction is subsequently applied to the protease strip/glass fibre filter combination. The results of this method of application of dust were adequate, an indication that an airborne collection device could be devised using a glass fibre filter pad through which a volume of air could be sucked.

The third method utilised a pad formed of protease membrane. This collection method gave good results, indicating that the collection method can be extremely straight forward, involving merely wiping the pad over a dusty surface.

In addition to the invention described hereinbefore it should be appreciated that the preceding description is not intended to limit the scope of the invention. It has also been found that apparatus incorporating a 3 electrode system is particularly preferable. The components of the apparatus and the levels of the various reagents, enzymes and catalysts used for this particularly preferred embodiment of the invention is described as follows:

Base electrode fabrication procedure

The three-electrode devices are fabricated by screen-printing using a DEK 248 printing machine. The apparatus consists of a three-electrode device incorporating a central circular working and semi-circular counter and

reference electrodes. Circular working electrodes allow reproducible droplet deposition of substances onto their surfaces, similarly, a circular disc cutter was available that allowed reproducible cutting of the membranes required 5 for functioning of the apparatus.

The base material for electrode fabrication consists of 250mm thick Polyester sheeting onto which are screen-printed 4 successive ink/paste layers. A first layer consists the underlying basal tracks and base working 10 electrode and counter electrode respectively, printed using MCA 45R carbon ink. A second layer, the circular working electrode electrocatalytic material, is an aqueous based ink prepared by thorough mixing of 1 part MCA 4A rhodinised carbon with 4 parts 2% hydroxyethyl cellulose in phosphate 15 buffer. A third layer utilises 15% silver chloride in silver paste to fabricate the reference electrode. In order to ensure that all 3 electrodes present a uniform planar surface area to the measurement surface and to protect the underlying basal tracks from the solution, an 20 epoxy based protective coating ink 242-SB (Agmet ESL Ltd., Reading, U. K.) is deposited (layer 4). The electrode apparatus is then heat treated for 2 hours at 120°C to cure the epoxy resin ink. The heating step also serves to stabilise the working electrode electrocatalytic material 25 such that it can withstand prolonged immersion in aqueous and mixed aqueous-organic solvent mixtures.

Reagents

Aqueous solutions are prepared in reverse osmosis water. L-amino acid oxidase (L-AAO) with a specific 30 activity of 0.55U mg⁻¹ is utilised (EC 1.4.3.2, extracted from diamond rattlesnake venom - *Crotalus adamanteus*).

Protease, from *Streptomyces griseus*, with a specific activity of 5.2 U mg⁻¹ is also used (Type XIV, EC 3.4.21.31).

35 Immunodyne ABC membranes, with a mean pore size of 3mm is used for both the blocking membrane and protease pad.

The Immunodyne membrane blocking solution incorporates 50mM N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) at pH7.8. The amino groups in the TES act to block sites that may encourage binding of free amino acid 5 constituents.

Amino acid oxidase biosensor

The underlying amino acid oxidase biosensors is prepared as follows: amino acid oxidase solutions (10ml volumes, 2.5U ml⁻¹ in 0.05M phosphate buffer, pH 7.8, total 10 L-AAO activity per electrode of 25mU) are pipetted onto the circular working electrode pads and allowed to dry for 1.5 hours prior to use. The sensors are stored at 4°C until required. When used under quiescent conditions, this format can be used for detecting liberated amino acids. However, 15 under vigorous stirred conditions an overlying membrane (e.g. cellulose acetate) is required to maintain the enzyme in close proximity to the transducer. All of the work described here was carried out using the apparatus under quiescent conditions.

Blocking membrane

As discussed above, a membrane is required in order to prevent contact between the two enzymes. Immunodyne membranes are cut to give circular discs, 14.5mm in diameter. Disks are cut using a rack and pinion lever press. This size of disk allows complete coverage of the portion of the 3 electrode screen-printed sensor not covered by the epoxy resin insulation layer. The membranes are blocked in a blocking solution (containing 50mM TES, 0.1M KCl and 0.01% Tween 20) under stirred conditions for 25 1 hour. After this time the membranes are removed and dried at room temperature, retaining the salts and detergent.

Protease pad

Immunodyne membranes are cut to give circular discs, 35 14.5 mm in diameter. Protease pads are prepared by depositing 20ml (657 U ml⁻¹) of the enzyme solution (prepared in 0.05M phosphate buffer pH 7.8) onto the

5 Immunodyne discs and leaving to dry, at ambient temperature, for 2 hours. After drying the discs are washed and blocked in 50mM TES solution, under stirred conditions, for 1 hour. Following this step, the discs are further washed in a solution containing 50mM TES, 0.1M KCl and 0.01% Tween 20 for 10 minutes. After this time the discs are left to dry at ambient temperature.

10 The working electrode is fabricated using a commercially available electrocatalyst (TMCA4a, MCA Services Ltd., Melbourn, Cambs., UK) that serves to reduce the potential at which the working electrode can be poised (in this case + 350 mV versus the silver/silver chloride reference electrode). This material has been well characterised in this laboratory (White et al., 1996, 15 1994a, 1994b). A degree of semi-selectivity is also given to the device by use of the electrocatalyst. The electrocatalyst is stabilised within the apparatus by cross-linking with hydroxyethyl cellulose (HEC) and heat treating for 2 hours at 120°C, the heating step also 20 serving to activate the catalyst. The HEC polymer allowed the material to be operated within aqueous phase environments.

25 The electrocatalyst/HEC complex forms a thick, porous pad that is ideal for immobilisation of biocomponents. L-amino acid oxidase is immobilised within this pad by physico-chemical means. Protease enzyme is covalently immobilised on pre-activated nylon-based membrane supports that are placed over the 3-electrode assembly.

30 A blocked membrane was placed between the two enzyme components in order to minimise the risk of L-AAO enzyme digestion by protease enzyme and also to minimise the adsorption of proteinaceous material to the electrode surface. Amino acid liberated by the protease digestion of protein within the dust sample diffuses across this spacer 35 membrane and is oxidised upon contacting immobilised L-AAO within the working electrode pad. The hydrogen peroxide

by-product of this enzymic reaction is quantified electrochemically as described above.

Apparatus assembly

The 'wipe' component

5 The protease pads are fixed to sheets of cleaned polyester (ICI Melinex type, Cadillac Plastics Ltd., Swindon, UK) by means of spray mount adhesive (3M, UK). The sheets were cut to yield single pad devices with dimensions of (1.4 cm x ~3 cm x 250 µm). This component of
10 the device is henceforth referred to as the 'wipe' as it is used to collect the dust sample for measurement

The 'electrode' component

15 Blank immunodyne pads are fixed to the L-AAO electrodes by spray mount adhesive (3M). Circular PVC discs (diameter 12.5 mm, cut using the Rack and Pinion Lever Press) and strips of PVC were used to shield the electrode and connector areas during adhesive addition. The blank pads are applied and pressed into place using cleaned polyester strips.

20 Test format

25 The test format consists of 6 separate stages, described in detail below. All tests are performed under ambient conditions. Tests are performed either in the laboratory with 'real' household dust samples or directly within the home environment.

1. The apparatus is removed from packaging and the two components (electrode and wipe) are separated. Care is taken not to touch either pad during the
30 measurement procedure.
2. The electrode component is dipped into a beaker of tap water so as to wet the blocking pad, whilst keeping the 3 electrical contact points dry. Excess water is gently shaken from the component. The electrode was then placed into the hand-held meter, pad side uppermost, such that the contact points slid into a connector apparatus.

3. The wipe component is dipped into a beaker of tap water so as to wet the protease pad, whilst keeping the 3 electrical contact points dry. Excess water is vigorously shaken from the component. The wipe is wiped across the surface to be tested, in a horizontal manner, in order to collect the sample and maximise the use of the protease pad area.
- 5
4. The wipe is then placed, pad down, on the electrode such that the wipe and electrode pads are in full contact and sandwiched together directly on top of each other. The 2 components are pressed together such that the residual spray mount gripped and held both parts.
- 10
5. The meter is switched on. The meter automatically monitors the electrochemical response of the assay for 7 min. At the end of the process the meter displayed the mean current response of the device over the last 3 minutes of the assay. The response is noted.
- 15
6. Where indicated, the test surface is cleaned with a commercially available cleaning product and stages 1-5 above are repeated with a new device. Cleaner surfaces will result in lower mean current response values.
- 20

Laboratory tests

25 All of the data unless specified otherwise has been conducted using the hand-held meter within a laboratory setting and provides ample evidence of the excellent performance characteristics of the 'metering apparatus'.

Home tests

30 Results are shown in the profiles below, the units being given in nano amperes.

PROFILE 1: 2 adults, 2 children, 0 pets

	<u>Before cleaning</u>	<u>After cleaning</u>
35 Wooden dressing table	171, 348	161, 193
Window sill of bedroom	191, 208	125, 76
Porcelain toilet top	484, 302	174, 222

27

Melamine kitchen work top	329, 656	141, 144
Metal fire hood, living room	291, 145	83, 91

PROFILE 2: 2 Adults, 0 children, 0 pets

5

Metal boiler top	307	172
Cupboard top	258	199

PROFILE 3: Office

10

Door ledge	1192	83
Desktop	149	112
Receptionists office	245	102

15 PROFILE 4: 1 Adult, 0 children, 0 pets

Kitchen floor	840
Mantelpiece	359
Spare bedroom	250
Computer table	270
Garage	220
Hall skirting board	272
HiFi Speaker	305
Cleaned surface	125

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PROFILE 5: 2 Adults, 0 children, 4 pets

Top of TV	255	101
Kitchen work surface	113	116

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A high degree of apparatus reliability is evident. Cleaned surfaces generally give significantly lower response values than non-cleaned surfaces. It is expected that the greater degree of apparatus uniformity which would be inherent in any controlled manufacturing process would increase device reliability still further.

Assay accuracy

The accuracy of the device for measurement of the protein levels in dust samples was compared with a standard spectrophotometric protein assay method. The performance 5 of the standard method was tested with a standard protein solution and suspensions of household dust samples to assess error due to dust sample inhomogeneity. The same dust suspensions were tested with the prototype apparatus to determine the difference in error between the two 10 methods. Finally, the whole prototype assay procedure was followed (dust collection by wet wipe) to determine the levels of sampling errors involved; accuracy was calculated by the percent bias method:

15 % bias = $\frac{\text{measured value} - \text{true value}}{\text{true value}} \times 100\%$

Percent bias is therefore a relative measure of how similar the measured value is to the true value. For example, if a 3 mg dust sample gives a measured response of 20 175 nA, whereas the calibration curve line of best fit predicts a value of 350 nA, then the bias value is -50%.

Calibration curves relating to the standard and prototype tests undertaken were prepared. The resultant 25 lines of best fit equations were used to calculate the 'true value' response of a given protein sample to each test method. The % bias of each individual measured data point could then be determined and a measure of assay accuracy obtained.

Dust measurement by standard spectrophotometric
30 protein assay

A standard household dust suspension was prepared in RO water and the protein content tested using the micro-BCA protein assay kit. The resultant calibration curve across 35 the dust range 0-1 mg is shown in Figure 5. Comparing the response values for dust and BSA, it can be seen that the dust used in this assay was approximately 10% protein. A good linear correlation was obtained ($R^2 = 0.984$). The

percent bias values of the standard protein measurement assay with dust suspension solutions across a range of dust quantities are shown in Figure 6.

Measurement of dust suspensions by apparatus

5 Dust suspensions across the range 0-1 mg were also tested using the prototype protein assay apparatus. 40 ml volumes of appropriately diluted dust stock solution were pipetted onto the device blank pads and the protease pad sandwiched on top. Results from the assay are shown in Figure 7,
10 which illustrates the calibration curve for the dust suspensions.

A general upward trend is seen in accordance with the quantity of dust sample applied and a clear distinction between samples containing <200 mg dust and ≥200 mg dust is
15 evident. The percent bias of the prototype device with the dust suspension samples is shown in Figure 8. 10 of the 20 bias values recorded were within 10% of the true value, similar to that obtained for dust suspensions tested with the spectrophotometric assay method.

20 Standard method versus prototype device

The performance of the 2 methods with respect to the quantification of protein in dust suspensions is compared in Figure 9 (sensor versus spectroscopic response actual data) and Figure 10 (sensor versus spectroscopic response background corrected data). The clear trends evident and the linear correlation data ($R^2 = 0.895$) confirm that a degree of correlation exists between the two systems.

Prototype device with dust collection by surface wiping

30 The response of the prototype device to different quantities of dry dust, collected by the protease pad wipe was assessed. Household dust samples were weighed out and dispersed on surfaces and collected. Responses are shown in Figure 11. This type of experiment measures the overall
35 degree of variability of the assay system - the non-homogeneous sample, device assay error and sampling error. A distinction between samples containing no dust and ≤ 1 mg

of dust is obvious using this system. Figure 12 illustrates the percent bias values of the prototype device with dust collection by wipe component across a range of dust quantities.

5 Assay precision

As with the accuracy tests, device precision was compared with a standard spectrophotometric protein assay method. The standard method was tested with a standard protein solution and household dust suspensions to assess 10 the precision errors due to dust sample inhomogeneity. The same dust suspensions were tested with the prototype device to determine the difference in precision between the two methods. Finally, the whole prototype assay procedure was examined (dust collection by wet wipe) to determine 15 precision with respect to the sampling operation.

The precision of the prototype apparatus was quantified by repeated testing the apparatus with known quantities of sample and calculating the standard deviation (SD) of the response values. SD values were standardised to 20 allow direct comparison by expression as percent coefficient of Variation (% CV), calculated as follows:

$$\text{CV (\%)} = 100\% \times \text{SD}/\text{mean current response}$$

25 Dust measurement by standard spectrophotometric protein assay

The % CV values for the measurement of household dust suspensions by the standard (spectrophotometric) assay method are shown in Table 2.

	Dust (mg)	Mean response value (A562)	% CV
5	0	0.081	24.3
	2	0.115	6.4
	5	0.154	9.4
	10	0.202	5.8
	15	0.260	17.1
	20	0.301	10.2
	40	0.465	12.3
	60	0.584	30.7
	80	0.669	12.0
	100	0.813	8.9

Table 2. Mean response and % CV values for assay of household dust suspensions by the standard (spectrophotometric) assay method.

Measurement of dust suspensions by prototype device

The % CV values for the measurement of household dust suspensions by the apparatus is shown in Table 3.

	Dust (mg)	Mean response value (nA)	% CV
20	0	161	17.9
	100	152	24.6
	200	212	22.4
	400	209	13.5
	600	265	9.1
	800	292	15.8
	1000	287	13.2

Table 3. Mean response and % CV values for assay of household dust suspensions by the apparatus.

The CV values of the assay process varied between 9.1-24.6 % with a mean value of 16.7%. This mean value compares favourably with the standard spectrophotometric protein assay where a mean value of 13.7% was obtained using equivalent dust suspensions. Thus, the prototype

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assay device has similar performance characteristics to the spectrophotometric approach with regard to precision.

Tests using household dust suspensions indicate that the apparatus, coupled to the developed hand-held meter, 5 has comparable performance characteristics to the industry standard spectrophotometric protein assay method. Approximately 50% of the dust suspension measurements had bias values within 10% of the expected value for both the standard measurement system and the apparatus. Similarly, 10 the standard and prototype assay methods exhibited similar precision profiles, yielding mean coefficient of variation (CV) values of 13.7% and 16.7% respectively. Further characterisation showed that the experimental error observed was mainly due to sample inhomogeneity rather than 15 the actual assay processes.

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CLAIMS

1. Apparatus which is a sensor for the detection of the level of protein in a sample and comprises:
 - a first reaction zone which contains a non-specific protease enzyme;
 - a second reaction zone containing a non-specific amino acid oxidase enzyme included at an electrode surface, and at least one other electrode which is capable of being in electrical contact with the first electrode;
 - a conduit for passage of liquid from the first reaction zone to the second reaction zone; and
 - separating means in the conduit for preventing passage of protease enzyme from the first reaction zone to the second reaction zone and for preventing passage of amino acid oxidase enzyme from the second reaction zone to the first reaction zone, said means allowing for the diffusion of amino acids.
2. Apparatus according to claim 1 in which the separating means comprises a chemically inert membrane having apertures of a size large enough to allow passage of low molecular weight compounds in solution but too small to allow passage of the high molecular weight compounds.
3. Apparatus according to claim 2 in which the low molecular weight compounds are amino acids and the high molecular weight compounds are enzymes.
4. Apparatus according to any of claims 1, 2 or 3 in which the protease enzyme is immobilised on a membrane, the apertures of which constitute the first reaction zone.
5. Apparatus according to any of claims 1 to 4 in which the amino acid oxidase enzyme is immobilised on a substrate.
- 35 6. Apparatus according to claim 5 in which the substrate is included in the catalytic surface of an electrode which catalyses the degradation of hydrogen

peroxide, preferably a transition metal catalyst, most preferably rhodium.

7. Apparatus according to any preceding claim in which the non-specific amino acid oxidase enzyme included in the electrode surface is in physical admixture with the catalyst.
5

8. Apparatus according to claim 7, in which the amino acid oxidase enzyme catalyst admixture is immobilised by provision of a protective coating which is
10 permeable to electrolyte and amino acids.

9. Apparatus according to any preceding claim, in which the first electrode is in liquid contact with the second reaction zone and the second electrode is in electrical contact with the first through an electrolyte
15 constituted by liquid in the second reaction zone;

10. Use of the apparatus as defined in any preceding claim for the detection of protein in a sample.

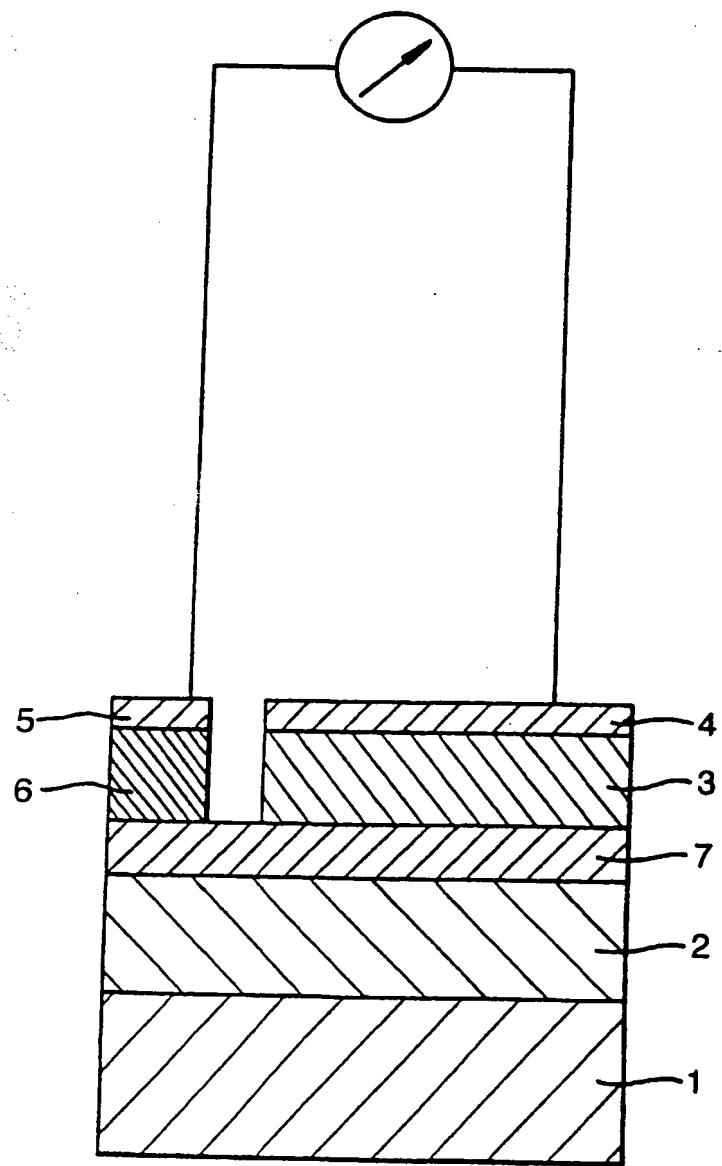
11. A kit comprising a plurality of components, wherein the components are suitable for constructing an
20 apparatus according to any of claims 1 to 9.

12. A kit according to claim 11, wherein the component constituting reaction zone 1 is presented separately of the component constituting reaction zone 2.

13. A kit according to claim 11 or claim 12,
25 wherein the components incorporate cooperation means.

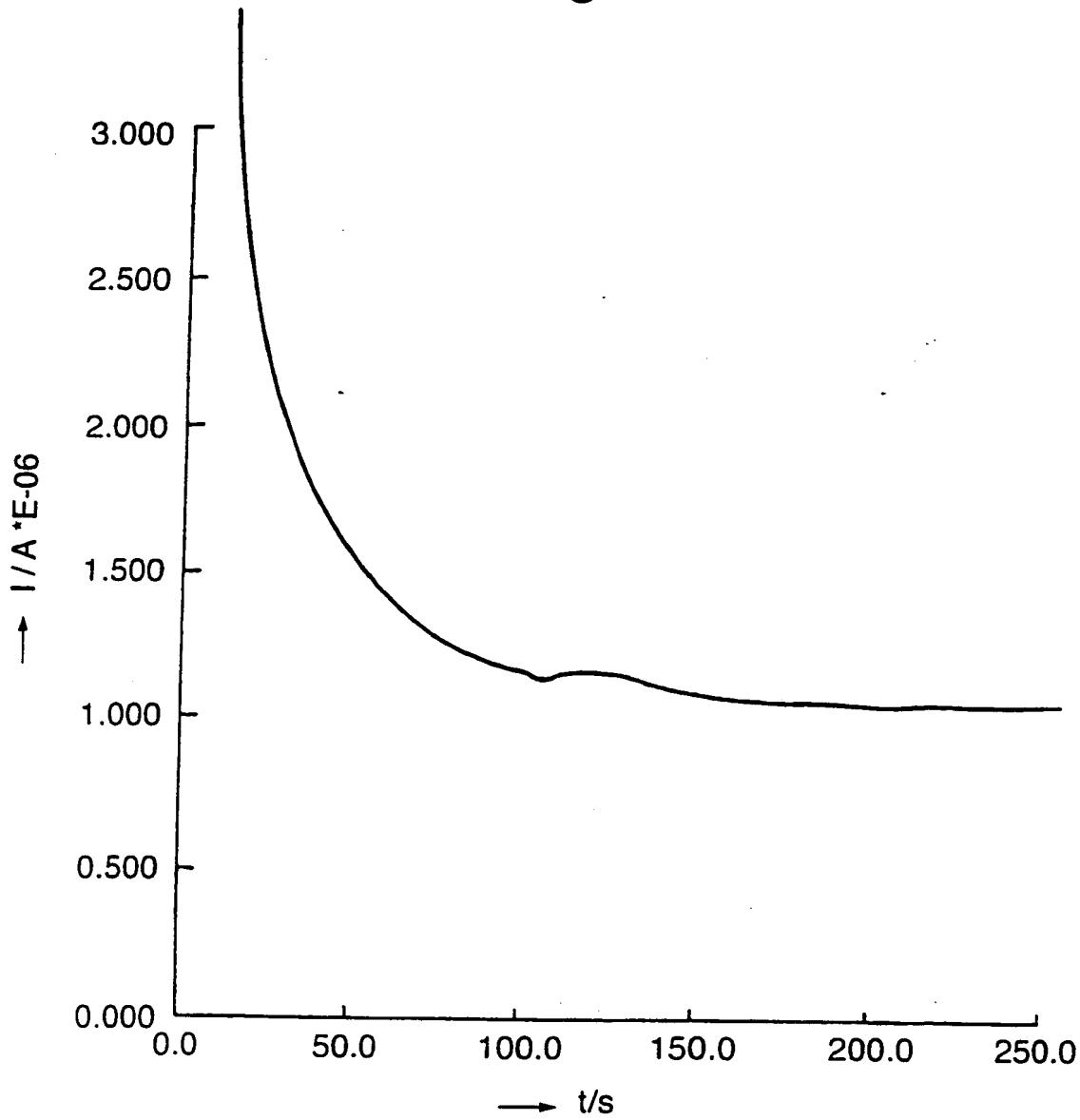
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Fig. 1.



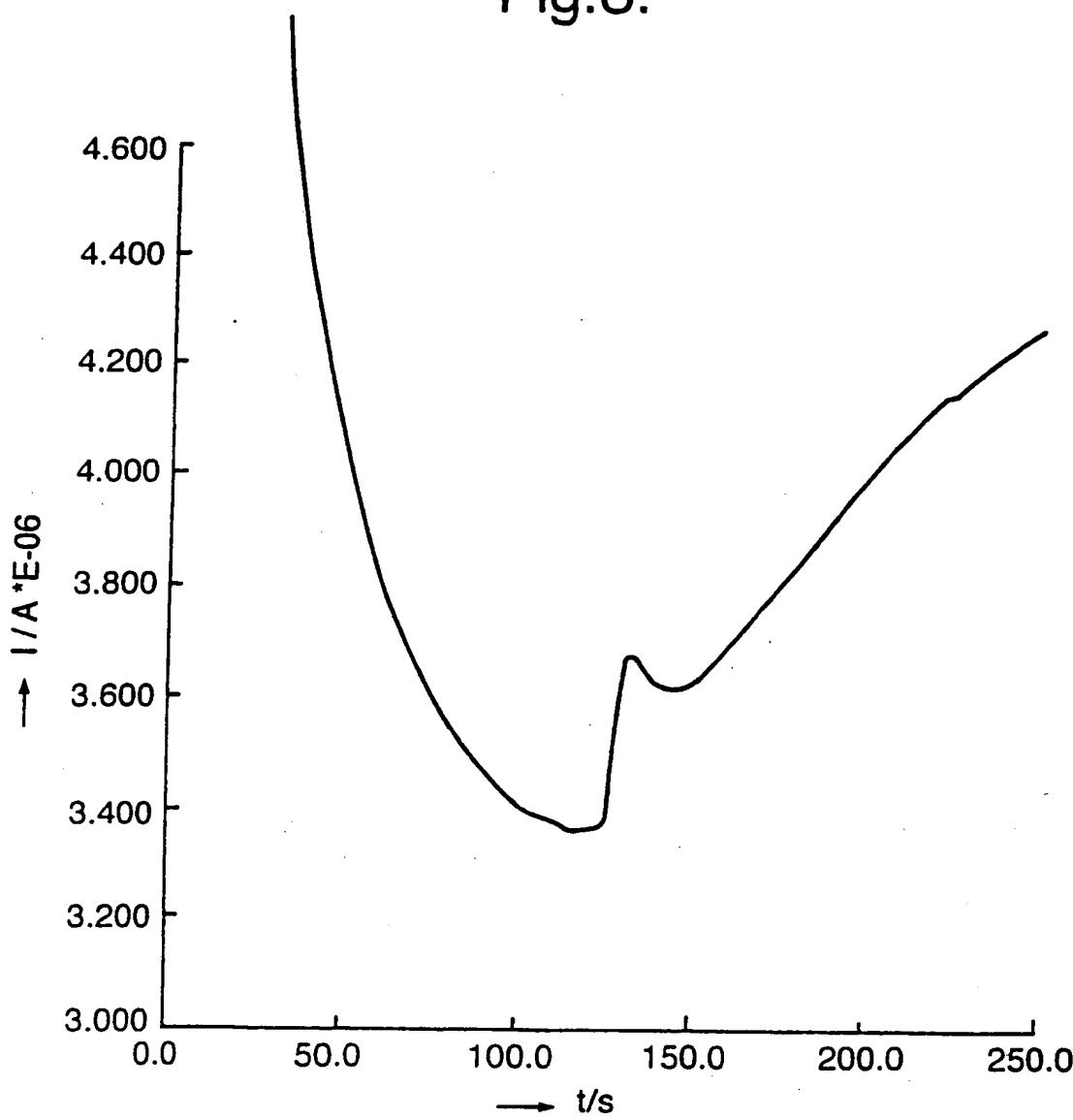
2/8

Fig.2.



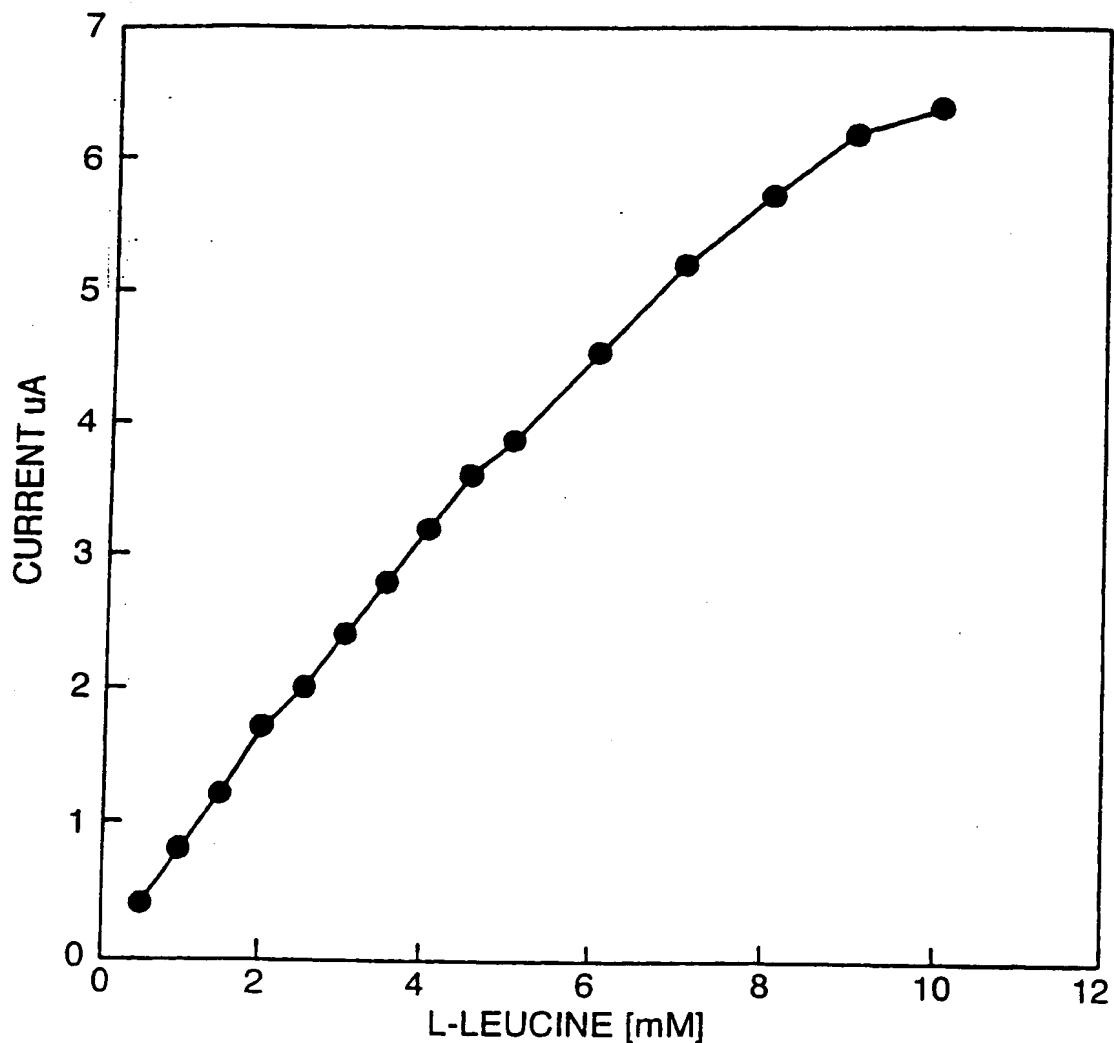
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Fig.3.



4 / 8

Fig.4.



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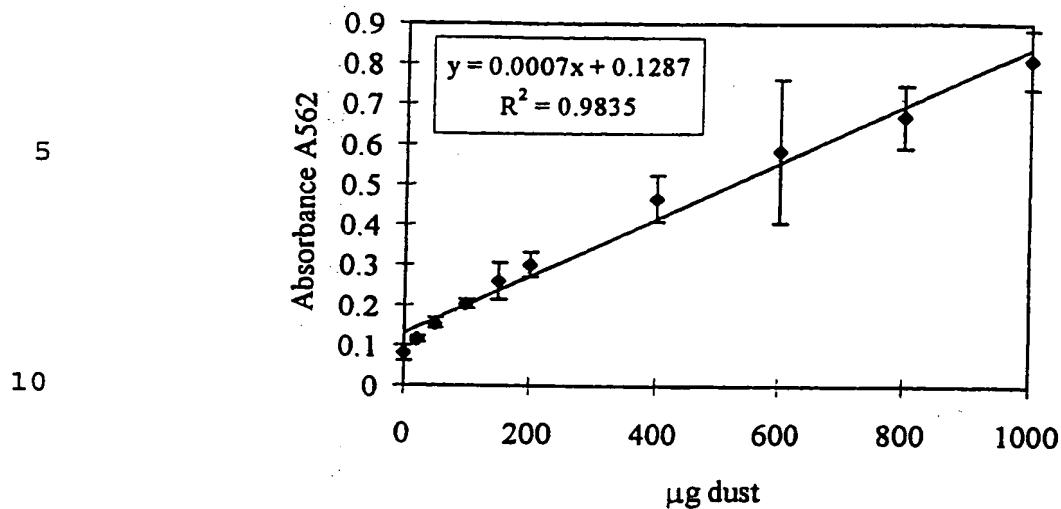


Figure 5.

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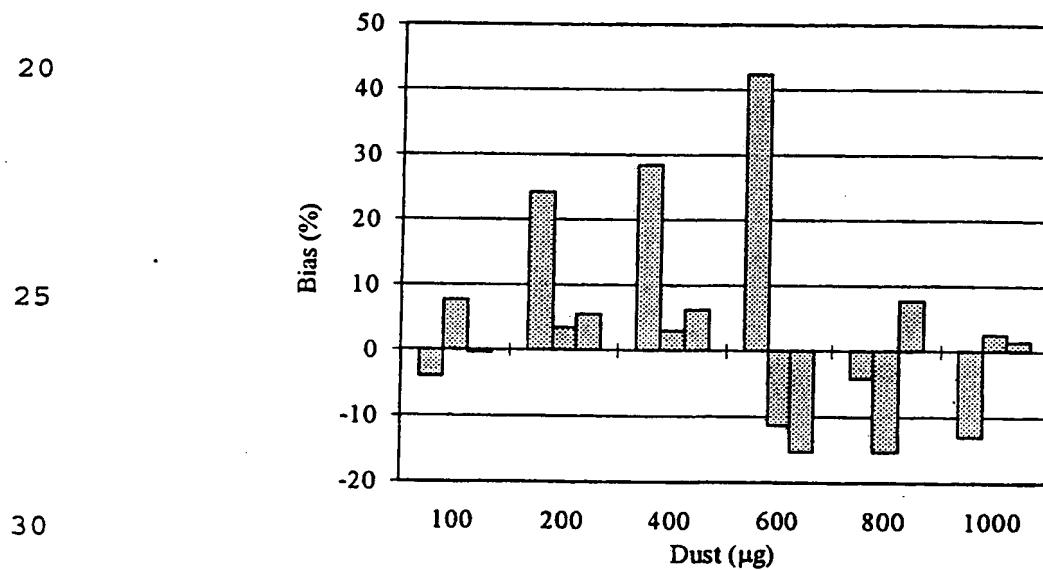


Figure 6.

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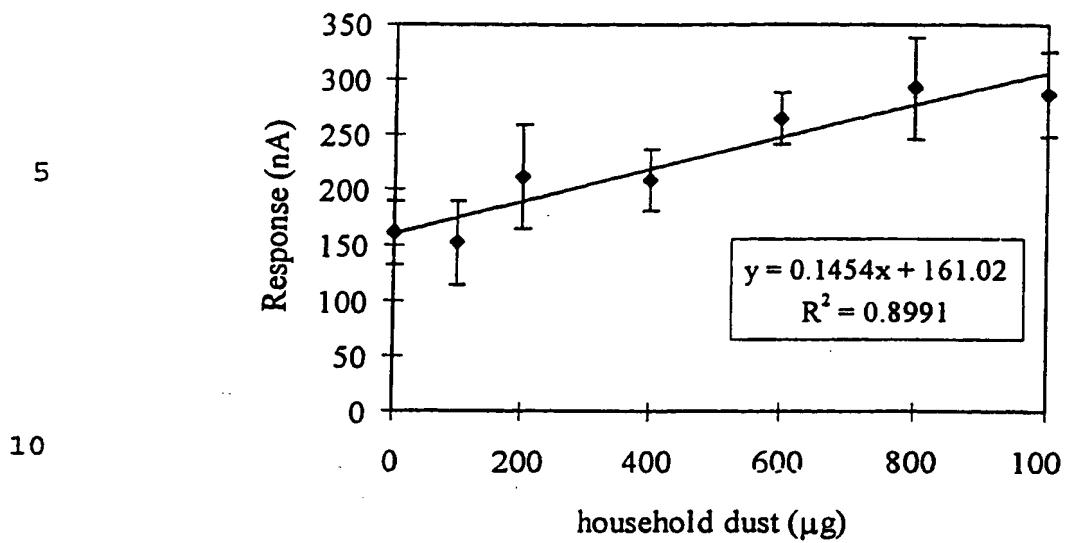


Figure 7.

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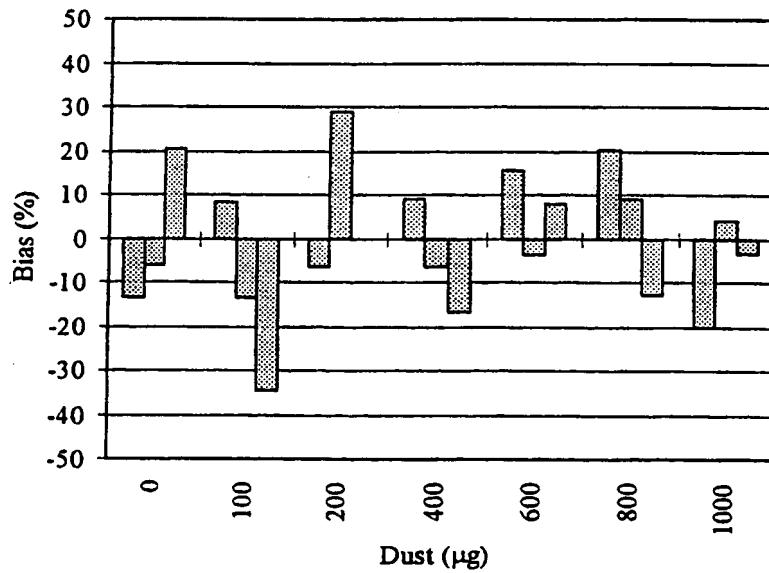


Figure 8.

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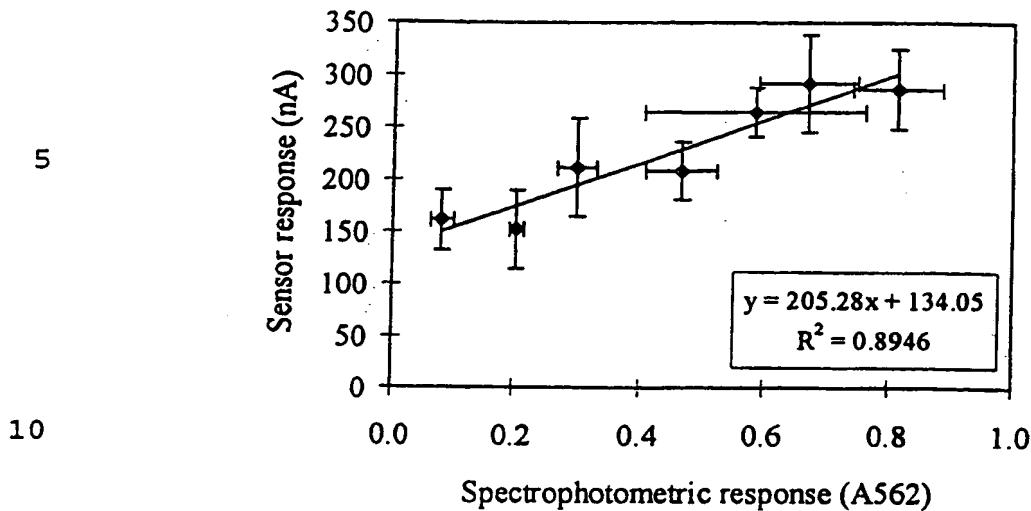


Figure 9.

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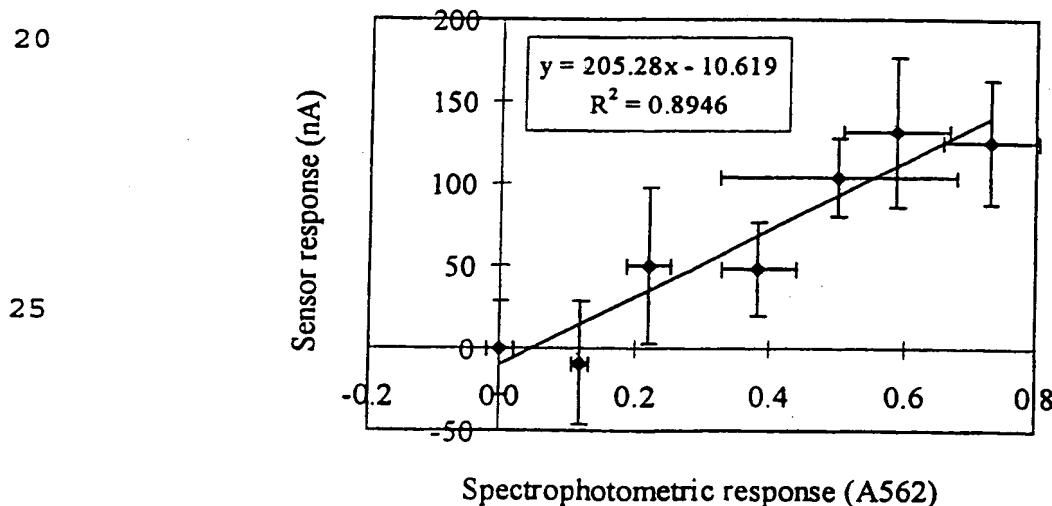


Figure 10.

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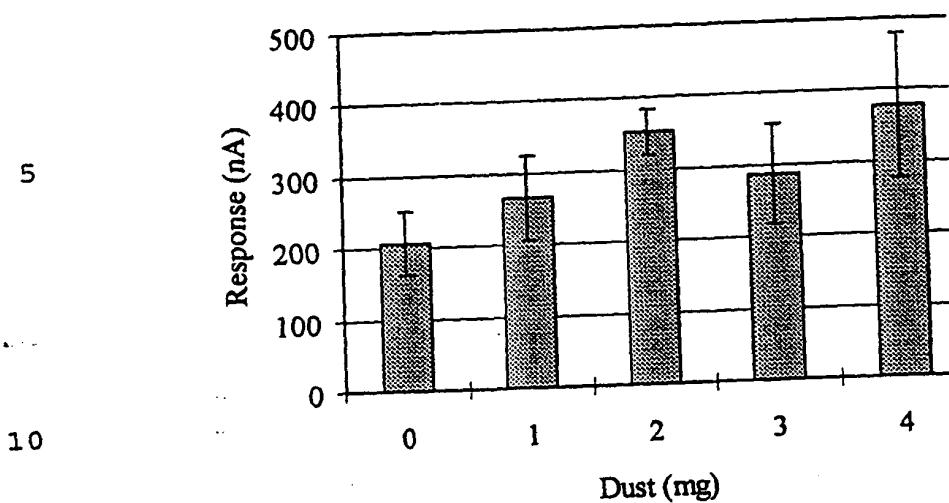


Figure 11.

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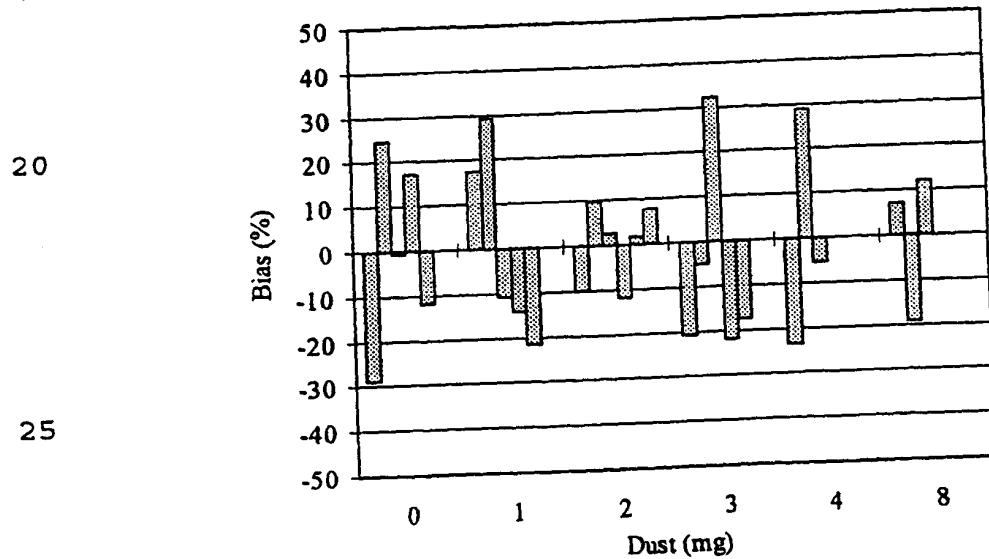


Figure 12.

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/02529

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 G01N27/327 C12Q1/00 C12Q1/37 C12Q1/26 C12N11/04

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE WPI Section Ch, Week 9303 Derwent Publications Ltd., London, GB; Class A89, AN 93-021929 XP002054643 & JP 04 348270 A (OKI ELECTRIC IND CO LTD) , 3 December 1992 see abstract</p> <p>---</p> <p>-/-</p>	1-13

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'E' earlier document but published on or after the international filing date
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- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
20 October 1998	06/11/1998
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Authorized officer Moreno, C

INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/GB 98/02529

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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